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Published in:
 Arthropod Structure & Development

DOI:
 [10.1016/j.asd.2007.09.003](https://doi.org/10.1016/j.asd.2007.09.003)

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Document Version
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Publication date:
 2008

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Citation for published version (APA):
 Giraldo, M. A., & Stavenga, D. G. (2008). Wing coloration and pigment gradients in scales of pierid butterflies. *Arthropod Structure & Development*, 37(2), 118-128. <https://doi.org/10.1016/j.asd.2007.09.003>

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Wing coloration and pigment gradients in scales of pierid butterflies

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Received 10 August 2007; accepted 19 September 2007

Abstract

Depending on the species, the individual scales of butterfly wings have a longitudinal gradient in structure and reflectance properties, as shown by scanning electron microscopy and microspectrophotometry. White scales of the male Small White, *Pieris rapae crucivora*, show a strong gradient in both the density in pigment granules and the reflectance. After pigment extraction by aqueous ammonia, scales of male *P. r. crucivora* closely resemble the unpigmented scales of female *P. r. crucivora*. Only a minor gradient exists in the white and orange scales of the male Orange Tip, *Anthocharis cardamines*. Pigment extraction of orange scales of *A. cardamines* causes bleaching. Partial bleaching transforms the scales so that they resemble certain scales of *Phoebis philea* that have a natural extreme gradient. Reflectance measurements on an artificial stack of two overlapping scales as well as on the scale stacks existing on intact and partially denuded wings of the Large White, *Pieris brassicae*, quantitatively demonstrate the reflectance enhancement by scale stacking.

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Keywords: Pterin pigments; Structural colour; Wing reflectance; Orange Tip

1. Introduction

Butterflies are among the most conspicuous animals, and their wing coloration is perhaps the most diverse in the animal kingdom. Considerable knowledge has been gained about the origin of butterfly wing patterns, which resemble pointillistic paintings where each point is formed by a coloured scale (Nijhout, 1991), but only recently has research become focused on the details of how single scales contribute to the global wing coloration (Vukusic et al., 1999; Yoshioka and Kinoshita, 2004; Stavenga et al., 2006; Giraldo and Stavenga, 2007).

A butterfly wing scale generally consists of two laminae, connected by trabeculae or pillars. The lower lamina is more or less flat and unstructured, but the upper lamina consists of densely spaced ridges, which are connected by crossribs. The area framed by adjacent ridges and crossribs is called a window (Ghiradella, 1998; Vukusic et al., 2000). Incident

light is scattered by the scale structures, because the refractive index of the scale material distinctly differs from that of air (Vukusic et al., 1999; Stavenga et al., 2004). The resulting scale's colour is either determined by its structural organization or its pigmentation, or by a combination of both properties (Vukusic and Sambles, 2003; Kinoshita and Yoshioka, 2005; Giraldo and Stavenga, 2007).

The colour of a whole butterfly wing is an even more complex phenomenon, because the scales are usually arranged in a system of distinct, partially overlapping rows of so-called cover and ground scales (Nijhout, 1991; Ghiradella, 1998). The wing substrate has in general two scale layers on both the dorsal and the ventral sides, and thus light reflected and transmitted by the five elements of the wing transect (including the wing membrane itself) determines the wing colour (Stavenga et al., 2006).

The optical and structural properties of single scales have been specifically studied in detail for scales featuring iridescent colours. Notably the tropical brilliant blue *Morpho* butterflies occupy many of the pages of the literature written so far about wing scales (e.g., Vukusic et al., 1999; Yoshioka and Kinoshita, 2004). The ridges of *Morpho* scales are elaborated

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into lamellae, which together form a multilayer where coherent scattering results in an intense blue reflectance. An ultraviolet version of the *Morpho* multilayer reflector is encountered in the dorsal cover scales of males of several pierid species, specifically of the subfamily Coliadinae. The coherent scattering of ultraviolet light by pierid scales is a sexually driven feature (Ghiradella et al., 1972; Silberglied and Taylor, 1973; Kemp et al., 2005; Rutowski et al., 2007).

The ground scales of male Coliadinae generally scatter light incoherently, as is the case in both cover and ground scales of female Coliadinae as well as of many members of the pierid subfamily Pierinae. In the present paper we focus on an important component of this scattering, one caused by ovoid-shaped granules that partially fill the scale windows, a microscopic characteristic only of the family Pieridae. These granules, also called beads, contain pigments that belong to the class of pterins. They execute a dual function. Depending on the type of pterin, they absorb light in the short-wavelength range, but outside the pigment absorption range, at the longer wavelengths, the granules strongly scatter light (Stavenga et al., 2004; Rutowski et al., 2005; Giraldo and Stavenga, 2007). The black and brown scales of pierids contain another type of pigment, melanin, which has a broad absorption spectrum, but these scales lack the beads, and hence the melanin is located in the ridges and/or crossribs (Yagi, 1954; Stavenga et al., 2004).

Microscopical observations of single scales reveal that the pigmentation is inhomogeneous. Here we report combined structural and optical studies on the scales of a number of pierid butterflies, and we detail how the gradient in pigmentation at the scale level will affect wing coloration.

2. Materials and methods

2.1. Animals

Japanese Small White butterflies, *Pieris rapae crucivora*, were obtained from Prof. K. Arikawa, University of Yokohama, Japan. The Orange Tip, *Anthocharis cardamines*, the Brimstone, *Gonepteryx rhamni*, and the Large White, *Pieris brassicae*, were collected in the Netherlands. The Orange-barred Sulphur, *Phoebis philea*, was obtained commercially.

2.2. Scale preparation and spectrophotometry

Single wing scales were isolated by gently pressing the wings to a glass plate and then were glued to the tip of a glass micropipette, which had a diameter of approximately 5 μm . Subsequently, the micropipette was mounted on a micromanipulator with one rotational and three translational degrees of freedom. For the experiments with overlapping scales, two micropipettes with scales were mounted on separate micromanipulators. Single scales were photographed with a Zeiss Axioskop microscope, applying bright-field epi-illumination or UV-induced fluorescence. Reflectance spectra were measured with a microspectrophotometer (MSP), which consisted of a xenon light source, a Leitz Ortholux microscope, and

a fibre optic spectrometer (SD2000, Avantes, Eerbeek, the Netherlands). The microscope objective was an Olympus 20 \times , NA 0.46. The reference was a white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA). The measurements on intact and partially denuded wings were performed with an integrating sphere and the fibre optic spectrometer, as described by Stavenga et al. (2006). The integrating sphere integrates the reflected light over the full 2π hemispherical angle, while the MSP objective integrates light over an angle limited by its aperture. The results of both methods are nevertheless directly comparable for pierid scales, because they act as Lambertian diffusers (Giraldo et al., in press).

2.3. Electron microscopy

After measuring reflectance spectra, the single scales were prepared for scanning electron microscopy (SEM) by sputtering the samples with palladium for 5 min with 800 V and 200 mTorr (Hummer, Technics, Alexandria, VA). The anatomy of the scales was investigated with a Philips XL-30, using a voltage of 3 kV. Small pieces of the forewing of a Brimstone, size about $1 \times 4 \text{ mm}^2$, were cut and processed for transmission electron microscopy (TEM). Samples were immersed in agar for better handling, prefixed in 2% glutaraldehyde/0.1 M Na-cacodylate and fixed in 1% OsO_4 /1.5% $\text{K}_4\text{Fe}(\text{CN})_6$ in 0.1 M cacodylate. Subsequent washing with double distilled water, and dehydration with an alcohol series that ended with 100%, were followed by propylene oxide for 30 min and embedding in Epon. Post-microtomed samples were contrast-enhanced with uranyl acetate in methanol for 2 min, lead-water for 1 min, and then examined with a Philips 201.

2.4. Bleaching scales

Drops of 1% aqueous ammonia were put locally on the wing in order to extract pterin pigment from the granules. The drops – with the extracted pigment – were taken away with filter paper after 10 min.

3. Results

3.1. Pigmentation of white scales of *P. r. crucivora*

In order to quantitatively compare the optical and anatomical properties of single scales, we have measured for each scale the reflectance of five areas (Fig. 1a), numbered from 1 to 5 from the base to the tip (Fig. 1b). Fig. 1 presents the case of a white cover scale from the dorsal wing of a male Small White, *P. r. crucivora*. The reflectance is minor in the near UV range, virtually independent of the location, but the reflectance is high in the visible wavelength range. The peak reflectance is about 15% in area 1 and gradually increases to almost 30% in area 5 (Fig. 1a).

Scanning electron microscopy (SEM) revealed a parallel increase in the density of beads, starting from a very low

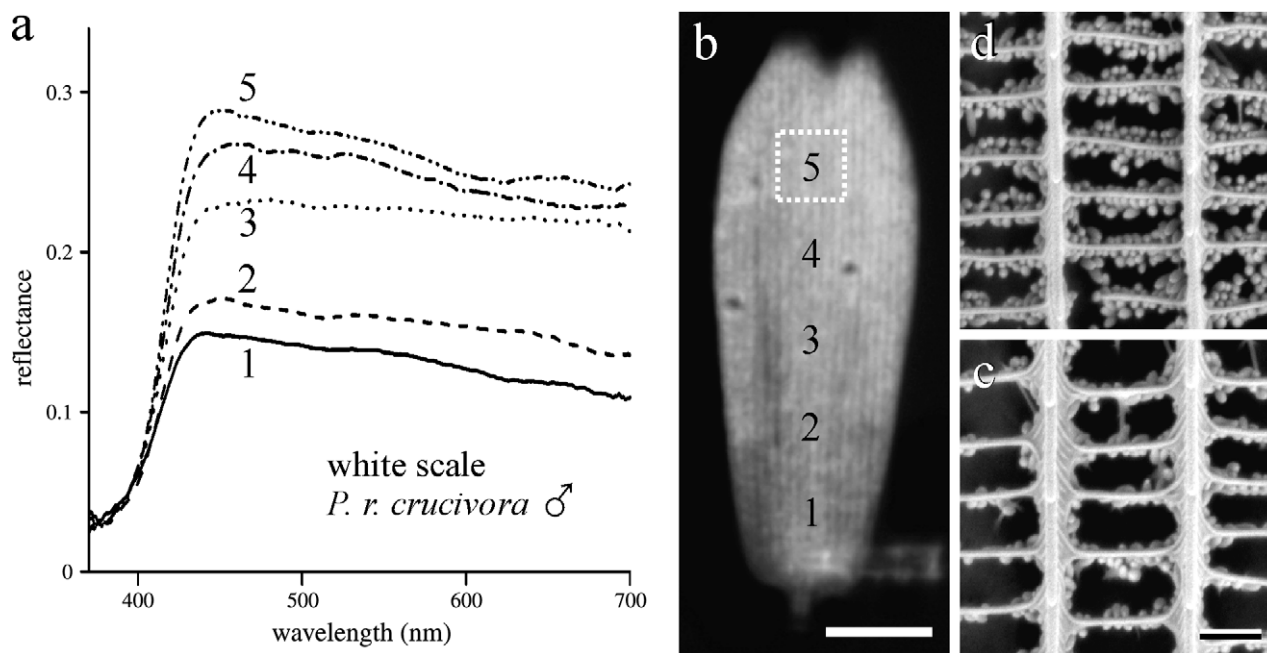


Fig. 1. (a) Reflectance spectra of a male *P. r. crucivora* of five different scale areas, numbered 1–5 from the base to the tip. (b) Reflection image of the scale photographed with an incident light microscope, with the area numbers. The dotted square represents the size of the measured area. (c, d) SEM images of the base area (1) and the tip area (5), respectively. The peak reflectance increases by almost a factor of two, when going from the base to the tip of the scale. The reflectance increase correlates well with the density of beads. Bars: 25 μm in (b), 1 μm in (c, d).

density in area 1 (Fig. 1c) to a high density in area 5, where the beads occupy most of the space in the windows, between the ridges and crossribs (Fig. 1d). We did not find a clear difference in the gradient of the bead density between cover and ground scales of *P. rapae*.

A comparative study of male and female *P. r. crucivora* (Giraldo and Stavenga, 2007) revealed that the white scales on the dorsal wing of female *P. r. crucivora* have a much higher reflectance in the UV and a much lower reflectance at the longer wavelengths than do the male scales (see also Figs. 2a, 1a). Both effects are a direct consequence of the very low density of beads in the female scales (Fig. 2b). The reflectance spectra are not completely flat, presumably due to some dependency of the scattering on the size of the ridge and crossrib structures.

Whereas female *P. r. crucivora* have a reduced number of beads in a natural way, beads can also be removed artificially, namely by applying to the wings aqueous ammonia, which extracts the pterin pigments that are concentrated in the beads (Kolyer and Reimschuessel, 1970; Morehouse et al., 2007; Wijnen et al., 2007). Scales of male *P. r. crucivora* wings treated with aqueous ammonia yield reflectance spectra with peak reflectances of less than 20% (Fig. 2c), very similar to those obtained from the female scales (Fig. 2a). SEM pictures indeed demonstrate that the scales of the ammonia-treated wing areas have lost the beads (Fig. 2d). The beads are not fully removed throughout the whole scale, however, as can be seen from the reflectance spectrum of area 1 (Fig. 2c), which features a low reflectance in the UV, and it is even more directly recognized from Fig. 2e, which is a photograph of the UV-induced blue fluorescence of a scale taken from an

ammonia-treated wing. The cuticle of the tip area (3–5) is distinctly fluorescing, which is not seen when the strongly UV-absorbing pterin pigment leucopterin (Wijnen et al., 2007) is present. The fluorescence is low in the base area (1, 2), because the excitation light is absorbed by the leucopterin that the ammonia has been unable to extract. This can be immediately understood, for the scales partly overlap each other, so that the base area is more or less protected.

3.2. Pigmentation of orange scales of *A. cardamines* and *P. philea*

The effect of pigment extraction on the reflectance spectra of pierid butterfly wing scales can be favourably investigated in the orange scales of the dorsal wing tip of the male Orange Tip, *A. cardamines*. Reflectance spectra measured from the various intact scale areas are very similar, with a low reflectance at wavelengths below 500 nm, and with a high reflectance above 600 nm, with peak reflectances of about 30% (Fig. 3a). The latter value indicates a high bead density throughout the scale, an anatomical property confirmed by SEM (not shown; also the white scales of male *A. cardamines* have a fairly constant and high bead density). In agreement with the reflectance spectra and the SEM photographs, scales taken from the dorsal wing tip of a male Orange Tip normally are orange throughout the scale, but scales taken from a wing treated with ammonia very clearly show a partial bleaching, that is, a partial extraction of the short-wavelength absorbing pigment (Fig. 3b). The reflectance spectra of the partially bleached scales give a more refined picture. Reflectance spectrum 1 of Fig. 3c is almost identical to spectrum 1 of Fig. 3a,

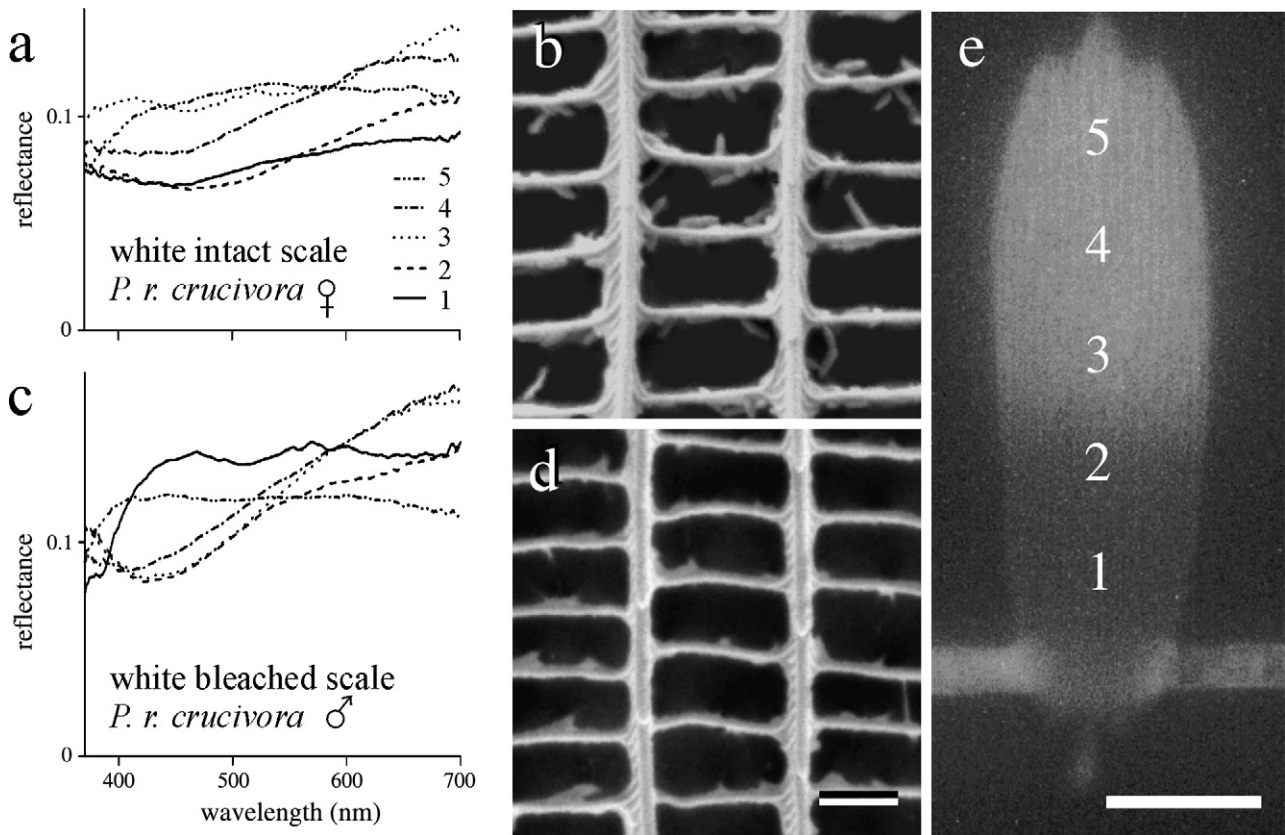


Fig. 2. Reflectance spectra and images of an intact female scale and of a bleached scale of a male *P. r. crucivora*. (a) The spectra for the five areas of a female scale from the base to the tip (1–5) do not reveal a clear gradient as in the case of the male, and the reflectance is much lower than that of a male scale (Fig. 1), which is due to the virtual lack of beads throughout the scale (b). (c) Reflectance spectra of a male scale where the pigmented beads were extracted with aqueous ammonia. The 10–15% peak reflectance is similar to that of the female scale. Spectrum number 1 corresponds to the base of the scale, which is not totally bleached due to the procedure used (see Section 2.4, Materials and methods). (d) The beads elsewhere vanished and the residual scale thus resembles a female scale. (e) UV-induced blue fluorescence photograph of a partly bleached scale of a male *P. r. crucivora* showing a low fluorescence in the base areas (1, 2), where pigment absorption is still present, and a high fluorescence in the tip areas (3–5), where pigment bleaching occurred. Bars: 1 μm in (b, d), 25 μm in (e).

with a low reflectance in the UV and a peak reflectance approaching 30%, and hence the ammonia has hardly affected the base area. The spectra 2–5 progressively deviate from spectrum 1, however, and the reflectance at scale area 5 is flattened to a virtually constant 17% for all wavelengths. SEM pictures are in complete agreement with the spectral data. In the strongly bleached scale tip area, beads are absent (Fig. 3d), while in the base area beads are plentiful (Fig. 3e).

The spectra of Fig. 3c clearly demonstrate the two opposite optical effects of the beads on the scale reflectance. In the absence of beads, the remaining scale elements, that is, the ridges and crossribs of the upper lamina and the lower lamina, together cause a rather wavelength-independent reflectance of about 17%. An increase in bead number means an enhanced pigment absorption, and therefore a reduced reflectance in the short-wavelength range, which is accompanied by an enhanced scattering in the long-wavelength range.

A similar clear demonstration of the dual role of beads is encountered in a special type of scale of the female Orange-barred Sulphur, *P. philea*. The ventral hindwings of the female *P. philea* are yellow with two bright white spots surrounded by orange-reddish rings. The white spot is created by

unpigmented scales that strongly reflect at all wavelengths, throughout the visible as well as the ultraviolet. The orange-reddish ring is formed by scales that have an extreme colour gradient that runs from white to orange-red, similar to the gradient artificially created in the orange scales of *A. cardamines* (Figs. 3b, 4). The reflectance spectra of Fig. 4a correspond to the five scale areas indicated in Fig. 4b. Spectrum 1, from the white area, is rather flat except for a slight peak around 410 nm. Presumably this peak is due to the lower lamina, which often acts as a thin film, a phenomenon frequently observed in white and coloured scales of many butterflies. Spectrum 5, from the orange-red tip of the scale, has a peak reflectance value of only 21%, much less than the 37% peak reflectance of the male Orange Tip scale (Fig. 3a). The short-wavelength reflectance is quite low, about 5%, somewhat higher than that of the orange scale of the male *A. cardamines* (Fig. 3a), also indicating a lower bead density of the *P. philea* scale.

The structure of the *P. philea* scales indeed deviates slightly from that of the scales of *P. r. crucivora* and *A. cardamines*. The crossribs are less sharply defined, and the windows are not filled with numerous beads. The orange-red and white scale areas

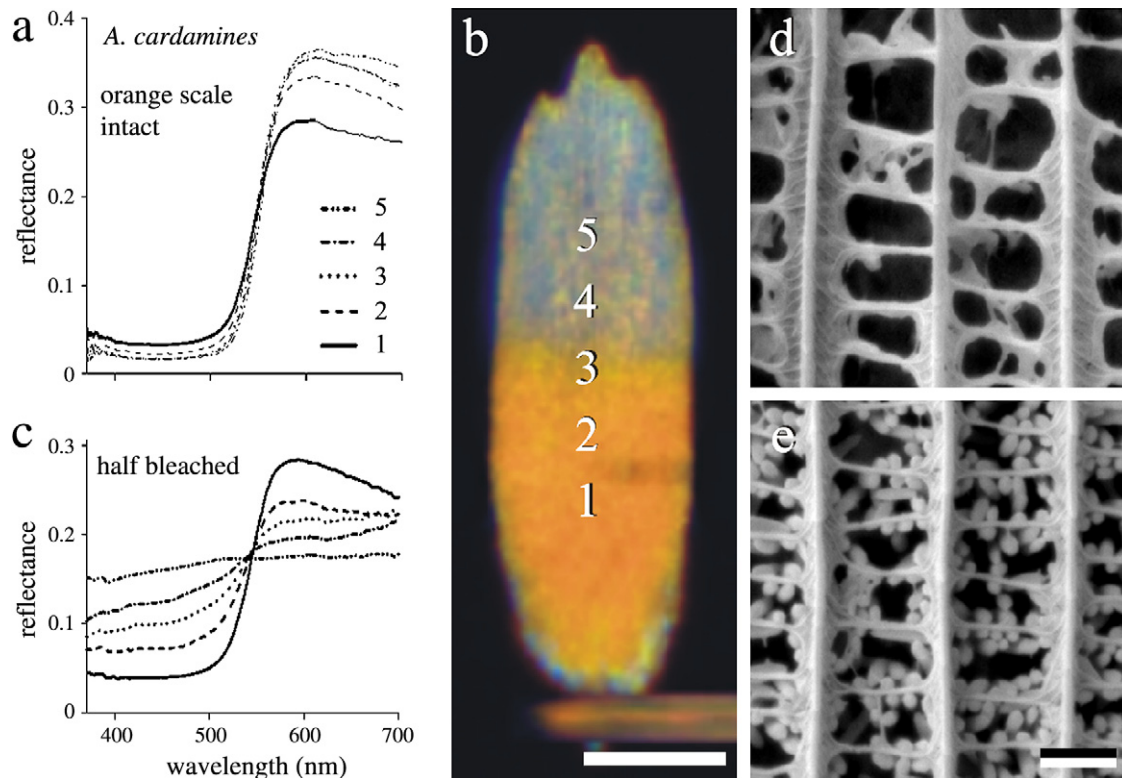


Fig. 3. Reflectance spectra of five scale areas of an intact (a) and half-bleached (c) orange scale of the male Orange Tip, *A. cardamines*. (b) Half-bleached scale with numbers indicating the five areas where the spectra of (c) were taken. The reflectance spectra of the intact scale reveal a gradient in long wavelengths scattering that, although important, is considerably less than that of *P. r. rapae* scales (Fig. 1a). Compared to the unbleached area 1, scattering as well as absorption is reduced in the strongly ammonia affected area 5. (d, e) SEM images of the bleached and unbleached scale areas. Bars: (b) 25 µm, (d, e) 1 µm.

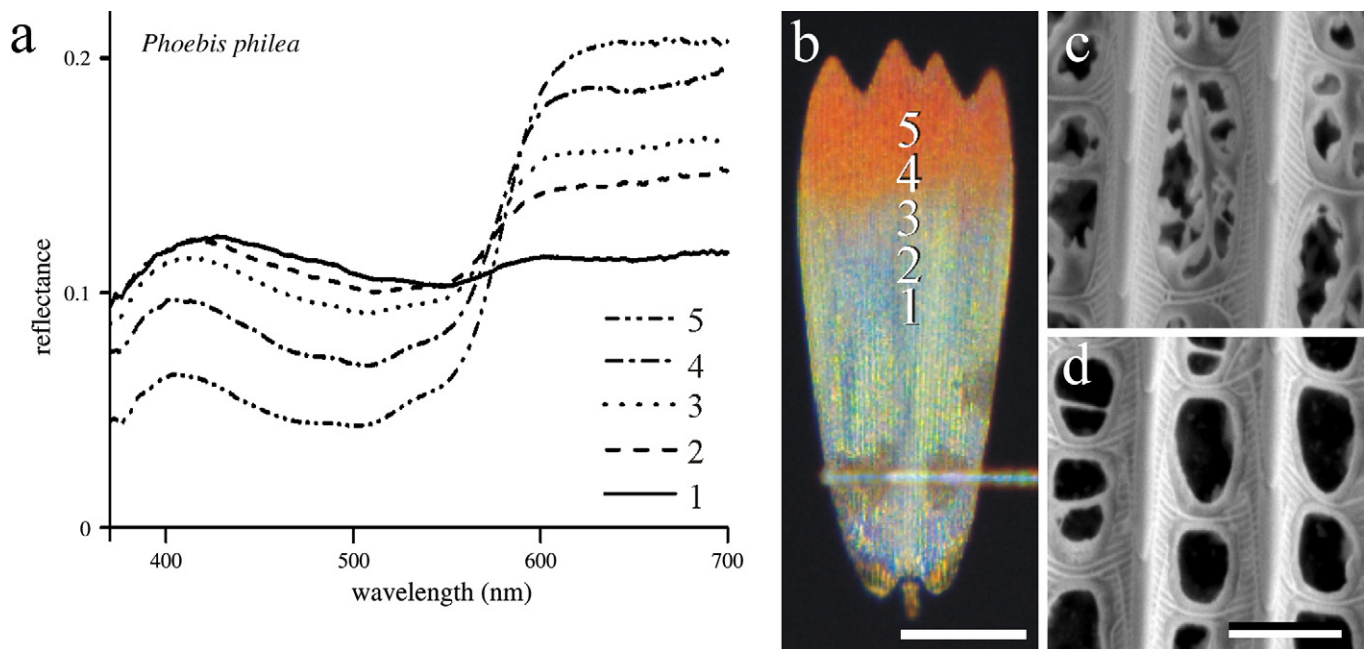


Fig. 4. A natural, partially coloured scale of a female *Phoebis philea*. (a) Reflectance spectra of five areas along the scale, which is shown in (b) glued to the tip of a glass micropipette; the numbers correspond to the five areas studied. (c) SEM image of an orange-red area, and (d) of a white area. The reflectance in the white area is rather constant, around 12%. Bars: (b) 50 µm, (c, d) 1 µm.

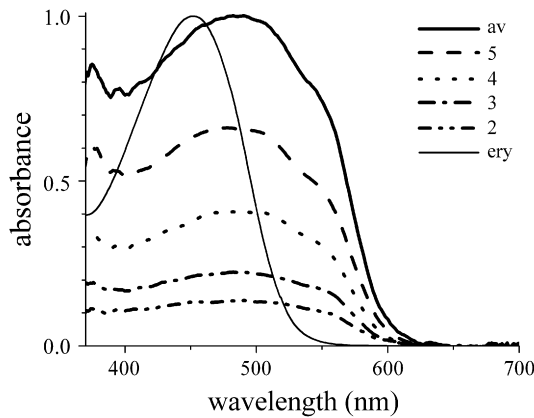


Fig. 5. Absorbance difference spectra (2–5) of the orange-red scale of *Phoebis philea* (Fig. 4b), calculated from the spectra of Fig. 4a (see text). The numbers refer to the scale locations of Fig. 4b. Normalization of spectra 2–5 with subsequent averaging yielded the pigment absorption spectrum (av). The absorption spectrum of erythropterin (ery) is given for comparison (from Wijnen et al., 2007).

clearly differ, as scale area 5 (Fig. 4c) has rather closed windows and area 1 (Fig. 4d) has open windows. The unpigmented area 1 has a virtually constant, wavelength-independent reflectance, suggesting that both the progressive decrease in reflectance at short wavelengths and the simultaneous progressive increase in reflectance at the longer wavelengths, when going from area 1 to 5, are proportional to the bead density. We have therefore further analyzed the pigmentation of the *P. philea* scale as follows. We argued that the reduction in short-wavelength reflectance with respect to the long-wavelength reflectance must be caused by an absorbing pigment that selectively absorbs light traveling through the scale and is only partly leaving the scale again as back-scattered light. To derive the absorption spectrum of the pigment, we modified a classical analysis method for pigments in complex media, namely to calculate absorbance difference spectra, i.e., the spectral differences between the $-\log_{10}$ of

the transmittance measured in states with different pigment concentrations. For each location 1–5 of Fig. 4, we have calculated a modified absorbance, by taking the $-\log_{10}$ of the reflectance spectrum with respect to the long-wavelength reflectance (taken as the average reflectance between 620 and 700 nm). We subsequently subtracted absorbance spectrum 1 from the other absorbance spectra, assuming that pigment absorption in location 1 was negligible. We thus obtained four absorbance difference spectra (2–5), presented in Fig. 5. The spectra appeared to be perfectly proportional to each other, suggesting that the magnitudes are proportional to the density of pigmented beads. To obtain the pigment absorption spectrum, we first normalized the four absorbance difference spectra of Fig. 5, and then we calculated the average (av, Fig. 5). For comparison, we added the absorption spectrum of erythropterin, the pterin extracted from orange and red coloured wings (Fig. 5: ery; from Wijnen et al., 2007). If indeed erythropterin is the pterin pigment of the orange-red scale of *P. philea*, then the absorption spectra of the pigment in situ (av) and in solution (ery) rather deviate, an observation also made on extractions of intact wings by Wijnen et al. (2007).

3.3. Optics of pierid scales

The structural and optical observations on pierid scales presented above can be summarized with a simplified two-layer model for the light flux in a scale (Fig. 6). Fig. 6a is a transmission electron microscopic section of a typical pierid wing scale (a ground scale of the dorsal wing of a male Brimstone, *G. rhamni*). Incident light is partially reflected (back-scattered) and transmitted (forward-scattered) by the structures of the upper lamina of the scales; that is, by the ridges, crossribs and beads. The transmitted light is in turn partially reflected and transmitted at the lower lamina of the scale. The light reflected at the lower lamina is subsequently partially reflected and transmitted at the upper lamina, and so on (Fig. 6a). The

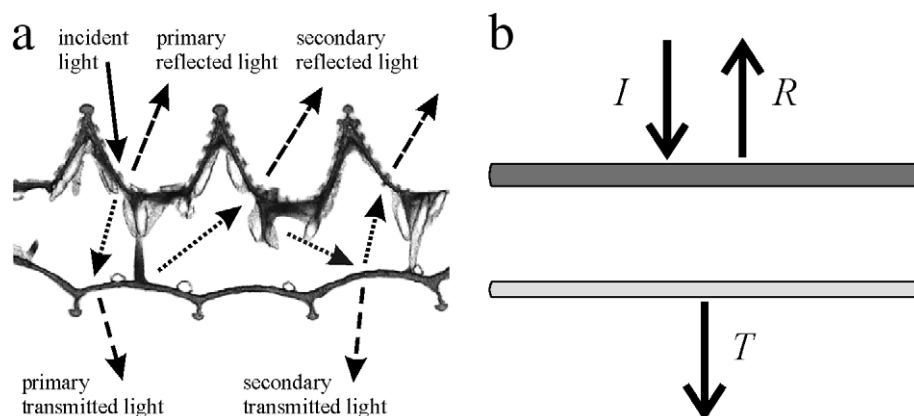


Fig. 6. Transmission electron microscopic image of a ground scale of the Brimstone, *Gonepteryx rhamni* (Pieridae, Coliadinae) with schematic indications of the light flux in the scale. (a) The dashed arrows represent possible trajectories of the light scattered by the scale structures. Incident light is partially back-scattered by ridges, crossribs and beads located in the upper lamina. The forward-scattered light is partly reflected by the smooth lower lamina, and then scattered again by the upper lamina structures, both backward and forward. In this way, the light reflected by the lower leaf has a second chance to be absorbed by the pigmented beads, and this process is repeated numerous times. (b) The scale reflectance, R , which is the fraction of the incident light flux (solid arrow) that leaves the upper lamina in the upward direction, is the sum total of the backward scattered light fraction, and the scale transmittance, T , is the sum total of the forward-scattered light fraction that leaves the lower lamina in the downward direction.

resulting scale reflectance (or transmittance), which is the fraction of the incident light that is reflected (transmitted), hence is the sum total of the primary, secondary, etc., reflected (transmitted) light fractions (Fig. 6b). We have to note here, of course, that the upper lamina of a scale is not a continuous layer, especially when the scale has large open windows and when the bead density is low (Figs. 1c, 2b, d). A considerable fraction of the incident light then will bypass the upper lamina structures and arrive undiminished at the lower lamina. Part of the light reflected by the lower lamina can similarly pass the upper lamina through the windows. Scales with few beads, and thus little absorbing pigment, will then yield a rather flat reflectance spectrum. However, with a high bead density, the windows are largely beset by the pigmented beads, and thus with a high absorption by the beads in the short-wavelength range and a strong light scattering in the long wavelength

range, a low reflectance at short wavelengths and a high reflectance at long wavelengths results.

A single scale can be treated as consisting of two layers, but effectively it acts as a single layer with reflectance (R) and transmittance (T), as indicated in Fig. 6b. The intact wing similarly can be considered as a single layer, because the scales on the intact wing overlap, forming scale stacks, and thus they determine, together with the wing membrane, the overall wing reflectance and transmittance. In Section 3.4 we present measurements of the wing reflectance and transmittance of the Large White, *P. brassicae*, but first we will discuss the reflectance of the most simple scale stack, consisting of two scales (Fig. 7). Fig. 7a and b present the reflectance spectra measured from the usual locations (1–5, see Fig. 1b) of two white scales (A and B) isolated from the dorsal wing of a Large White. The two spectral sets are, of course, not identical, but they are very

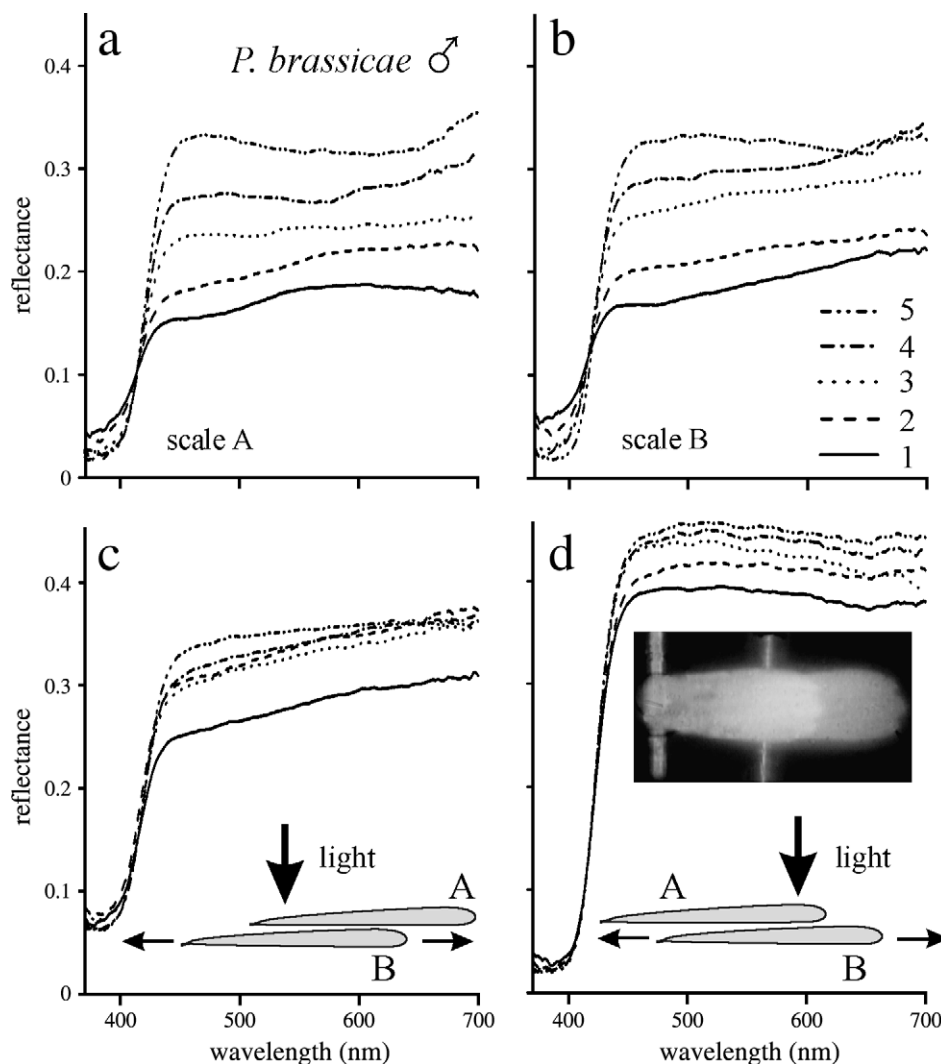


Fig. 7. Reflectance spectra of two scales of a male *P. brassicae*. (a) Spectra of isolated scale A and (b) of isolated scale B. (c) Spectra of the base of scale A (location 1) over five areas of scale B (locations 1–5; see Fig. 1b). (d) Spectra of the tip of scale A (location 5) over five areas of scale B (locations 1–5). Overlapping scales have a higher reflectance than the isolated, single scales. The photograph inset shows the two overlapping scales, in a position approximating that in situ, but here glued to micropipettes. Notice that the area of the superimposed scales looks whiter. The horizontal arrows in the inset diagrams indicate that scale B was moved in steps below a stable scale A.

similar, with a decreasing reflectance in the UV and an increasing reflectance in the visible range, when going from the scale base to the scale tip. To study the effect of overlap, we mounted the two microelectrodes with scales A and B on two separate micromanipulators, which allowed precise manipulation of the scales (inset photograph, Fig. 7d). The distance of the scale planes was ca. 10 μm , which is about the usual distance of

scales on the wing. In the experiment of Fig. 7c, scale B was moved in small steps, so that its locations 1–5 were directly underneath location 1 of scale A, and then the reflectance was measured in each of the five situations (see inset diagram). In the experiment of Fig. 7d, scale B was similarly moved in small steps, so that locations 1–5 were directly underneath location 5 of scale A. The vertical arrows in the inset diagrams of Fig. 7c

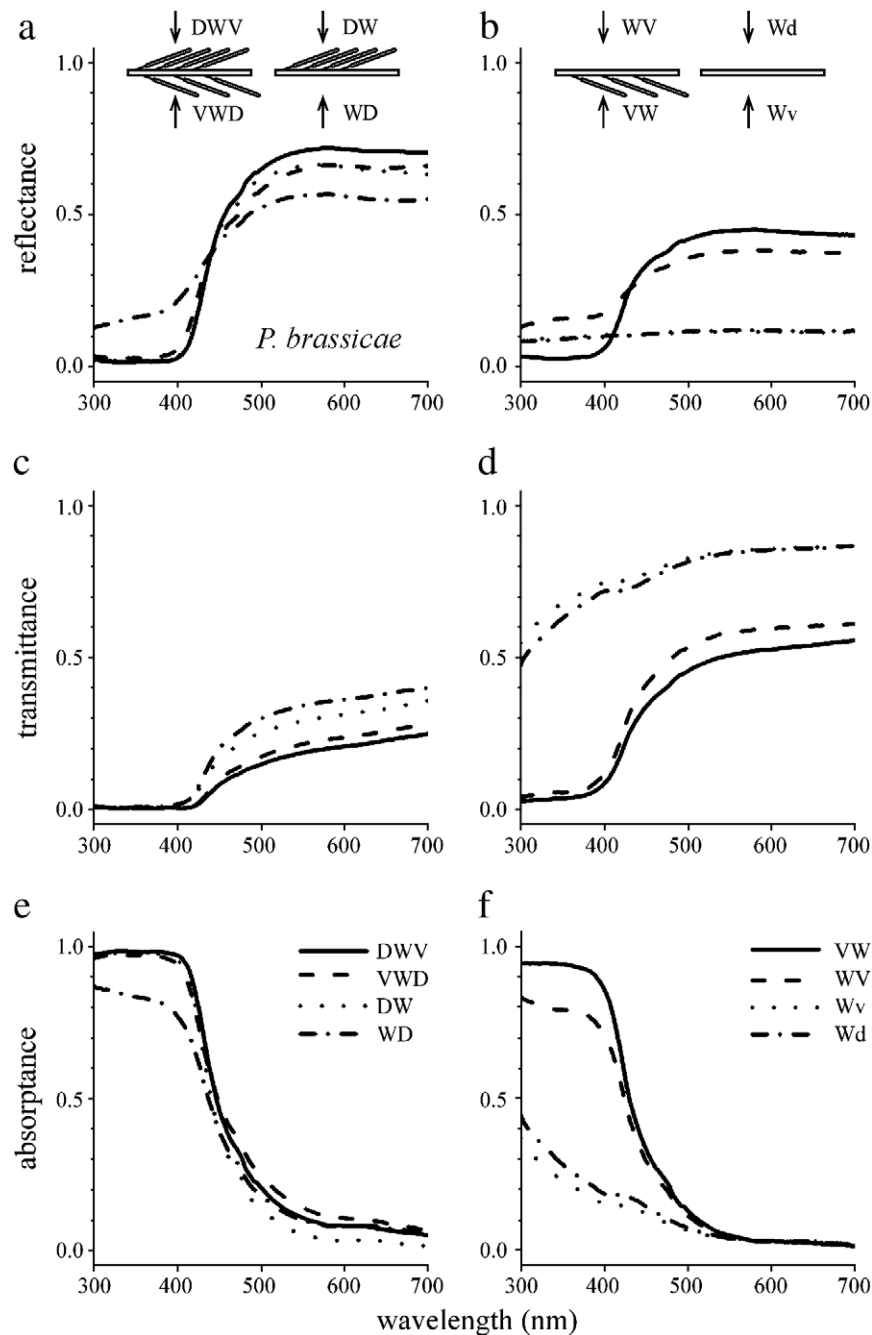


Fig. 8. Reflectance (a, b) and transmittance (c, d) spectra measured with an integrating sphere from forewings of the Large White butterfly, *Pieris brassicae*, in various conditions, together with the calculated absorbance spectra (e, f). The wing was intact for the conditions DWV and VWD, where D indicates the dorsal side of the wing, W is the wing substrate, and V is the ventral side; the order of the letters indicates the direction of the incident light. For DW and WD, the wing scales were removed from the ventral side, and for VW and WV, the wing scales were removed from the dorsal side. For Wv and Wd, both dorsal and ventral scales were removed, and the incident light came from the ventral (v) and dorsal side (d), respectively. The scales contain a strongly UV absorbing pigment, resulting in a very low transmittance and a very high absorbance in the ultraviolet. The wing scales strongly scatter in the visible wavelength range. The reflectance of the denuded wing is virtually constant throughout the whole spectral range (b), and the wing substrate contains a small amount of pigment that absorbs in the UV (f).

and d indicate the incident light beam. The reflectance spectra show that the reflectance in the basal area is about 18% for a single scale (Fig. 7a, b), which increases to maximally about 35% for a stack of two scales (Fig. 7c). The reflectance in the tip area is about 33% for a single scale (Fig. 7a, b) and increases to about 45% for a stack of two scales (Fig. 7d). This demonstrates that the enhancement of the reflectance by scale stacking strongly depends on the characteristics of the top layer. The long wavelength-reflectance values of Fig. 7d (tip of A on top) are all larger than those of Fig. 7c (base on top). This suggests that the function of the top position of the highly reflecting scale tips is to optimize wing reflectance.

3.4. Optics of pierid wings

In the native, intact wing situation, scale stacks exist on both sides of the wings. We previously performed a detailed analysis of the effect of scale stacking for the Small White, *P. rapae* (Stavenga et al., 2006). Here we present the same treatment for the Large White, *P. brassicae* (Fig. 8). Briefly, we used an integrating sphere to measure the reflectance, R (Fig. 8a, b), and transmittance, T (Fig. 8c, d), of intact and denuded forewings, and we then calculated the absorptance, that is the light fraction absorbed, with $A = 1 - R - T$ (Fig. 8e, f). The measurements of Fig. 8a show that the long-wavelength reflectance of the intact wing, using incident light from the dorsal side (DWV), is higher than that when the illumination is from the ventral side (VWD), thus revealing an asymmetry in the wing optics. Fig. 8c shows a similar asymmetry for the wing transmittance. A clear asymmetry also occurs when the scales on one

side of the wing are removed (for instance DW vs WD, Fig. 8a, c; or WV vs VW, Fig. 8b, d). The wing scales strongly absorb short-wavelength light, presumably due to the scale pigment leucopterin (Wijnen et al., 2007), and also the wing substrate appears to be slightly pigmented (Fig. 8f).

By considering the scale stack on the dorsal side (D), that on the ventral side (V), and the wing substrate (W) each as a separate layer, we can calculate the two opposite reflectances, r and s , as well as the two transmittances, t and u (Fig. 9b, inset), together with the absorptances, a , with the formalism of Stavenga et al. (2006). The reflectances in the visible wavelength range of the dorsal (r_D and s_D , Fig. 9a) and ventral (r_V and s_V , Fig. 9b) scale stacks were determined to be about 30–40%, similar to the measured reflectances of the artificial stacks of two scales of Fig. 7. Indeed, visual inspection shows that on the wings of *P. brassicae* in average about two scales overlap. The scale stacks on both sides of the wing, together with the wing substrate, result in a total reflectance of up to 70% (Fig. 8). As we have argued before, this will be about optimal, as a further increase in the number of overlapping scales will enhance the reflectance only to a minor extent (Stavenga et al., 2006).

A basic assumption of the applied model is that light scattering in the various layers is random (Stavenga et al., 2006). Direct measurements of scattering by single scales of the Small White, *P. rapae*, indicate that this assumption approximately holds (Giraldo et al., in press). A further check of the model can be performed by calculating the reflectance and the transmittance of the intact wing from the known reflectances and transmittances of the three wing layers (for procedure,

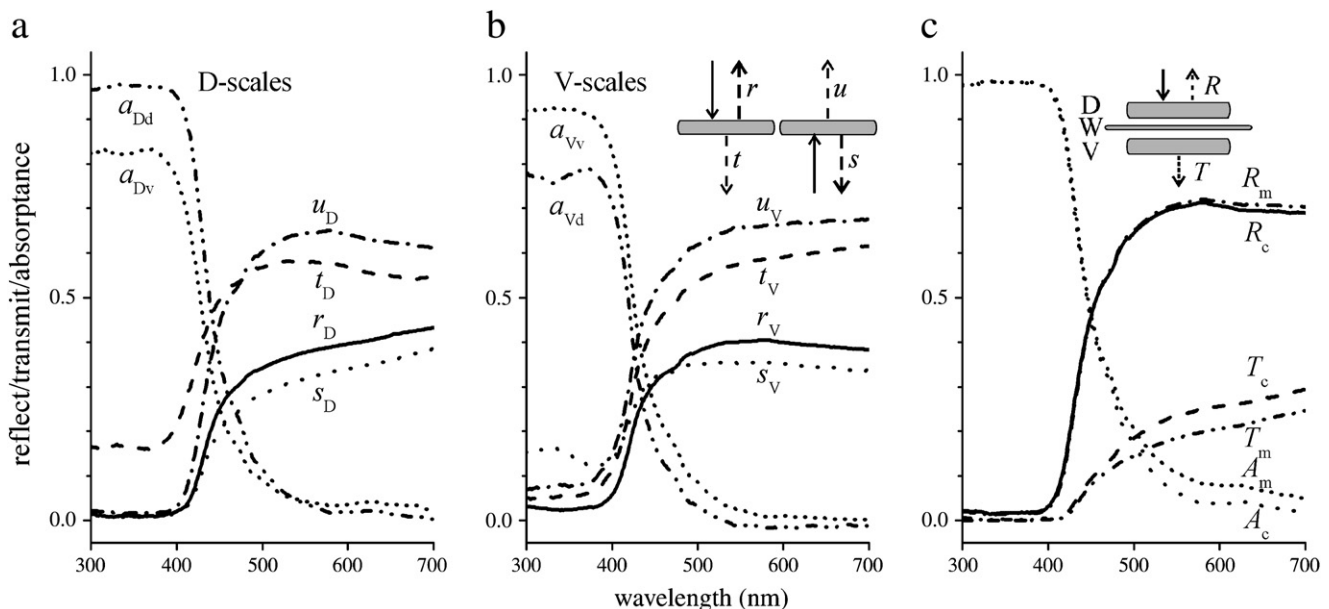


Fig. 9. Reflectances, r and s , and transmittances, t and u , of the dorsal (a, index D) and ventral (b, index V) scale layers of the dorsal forewing of *P. brassicae*, calculated with the data of Fig. 8 and the formalism of Stavenga et al. (2006). Inset in (b): reflectance r and transmittance t refer to incident light directed towards the wing substrate; reflectance s and transmittance u refer to incident light directed away from the wing substrate. The absorptance for the D-scales illuminated from the dorsal (d) side was calculated from $a_{Dd} = 1 - r_D - t_D$, and the absorptance with illumination from the ventral (v) side was calculated from $a_{Dv} = 1 - s_D - u_D$. (c) The reflectance and transmittance spectra measured from the intact wing with incident light from the dorsal side (DWV, see inset), R_m and T_m (continuous lines in Fig. 8a and c), compared with the spectra, R_c and T_c , calculated with the formalism of Stavenga et al. (2006). The calculated transmittance is slightly larger than the measured transmittance, and accordingly the calculated absorptances, calculated with $A_{m,c} = 1 - R_{m,c} - T_{m,c}$, slightly differ.

see Stavenga et al., 2006). The reflectance spectrum calculated for dorsally incident light (DWV, R_c) indeed well matches the measured reflectance spectrum (R_m , Fig. 9c), but the transmittances, and accordingly the absorptances, slightly differ. Scattering in the *Pieris* scales hence is approximately, but not perfectly random (Giraldo et al., in press). The absorptances indicate that ultraviolet incident light is virtually completely absorbed, and that the scales also absorb a substantial fraction of blue light, thus yielding the very slightly yellowish coloration of the Large White.

4. Discussion

The scales of pierids are unusual among the butterflies, because the pigmentation is localized to granular beads. The distribution of the beads in the pierid scales appears to vary strongly among species. For instance, the scales on the dorsal wings of female *P. r. crucivora* virtually lack beads, while the males have scales with a high concentration of beads, but with a distinct longitudinal gradient (Figs. 1, 2). The orange-reddish scales of *P. philea* have an extreme gradient, with only orange-red pigmentation in the scale tip (Fig. 4b).

Another variation in the scale structure is the shape of the windows, which in most pierids are wide open, well-defined by the ridges and the rather thin crossribs. In the *P. philea* scales, the windows are much less open and partly filled by a laminar membrane (Fig. 4c, d). Actually, quite frequently some membranous structure can be seen in the windows, as shown in Fig. 10, which presents scales of a male *P. r. crucivora* that are presumably arrested in various stages of development. In Fig. 10a, the upper lamina covers the windows while pigment granules (beads) are wrapped within the lamina membrane material. The beads are separated to various degrees in other scales, where they are connected to each other and to the crossribs by thin strands of membrane material (Fig. 10b–d).

The separation in numerous distinct beads is extreme in males of the Small White and Large White, and then the beads create extreme optical effects. The beads effectively absorb short-wavelength light, but at the same time strongly scatter long-wavelength light, thus distinctly whitening the wings (Stavenga et al., 2004; Morehouse et al., 2007). When the beads were embedded in a more or less continuous membrane, as in Fig. 10a, b, a much lower reflectance would result. By stacking the well-scattering scales in two overlapping layers

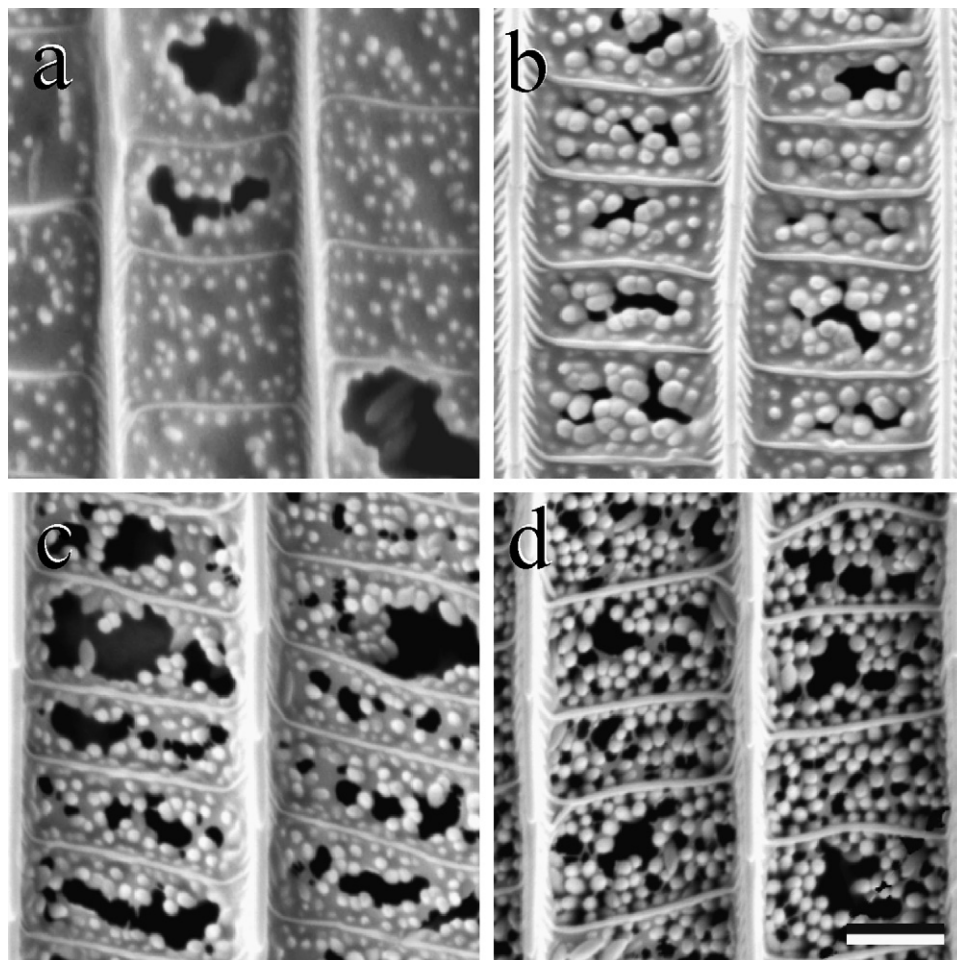


Fig. 10. Scales of *P. r. crucivora* arrested in different stages of development. (a) In the immature scales, a lamina covers the windows, and pigment granules (beads) are wrapped within the lamina material. (b–d) In more developed scales, the beads are becoming more and more separate, and connected only by thin strands of lamina material onto each other and to the crossribs. Bar: 1 μ m.

on each side of the wing, a very high wing reflectance of 60–70% is realized, a remarkable achievement given the minimal amount of material mass that is involved.

During development, butterfly wings generally have to specify several patterns at once, for instance the basic shape, venation patterning, deployment of scales, distribution of pigmentary and structural colours, the details of scale morphology. Our present findings show that additional details are the degree of pigment gradients and scale overlap.

We conclude from our combined structural and optical experiments, on single scales as well as on intact and denuded wings of pierid butterflies, that the non-iridescent coloration of pierid wings can be largely understood from the random light scattering in the overlapping layers of scales. Of course, there are several remaining questions, for instance about the development of the scales, about possible functional reasons for the variations in bead gradients among species, and about the chemical mechanisms that cause the deviant pterin absorption spectra *in situ* (Wijnen et al., 2007).

Acknowledgements

Prof. J.T.M. de Hosson and G. ten Brink (Materials Science Department, University of Groningen) provided essential support for scanning microscopy, and Dr. H. van der Want together with D. Kalicharan and H. Blaauw (Electron Microscopy department, Cellular Biology, University of Groningen) provided the facilities for the transmission electron microscopy. Prof. H. Ghiradella offered valuable criticisms to the manuscript. Financial support was given by the EOARD (Grant 063027).

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